Anti-Caspr-Conjugated Gold Nanoparticles Emergence as a Novel Approach in the Treatment of EAE Animal Model

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Research Article

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disorder of central nervous system which is increasing worldwide. Although immunosuppressive agents are used for the treatment of MS disease, nevertheless the lack of non-toxic and efficient therapeutic method is perceptible. Hence, this study aims to evaluate the effect of Contactin-associated protein (Caspr) antibody-, poly ethylene glycol (PEG)- and exosome combined gold nanoparticles (GNPs) in comparison to Glatiramer acetate as a selective treatment of MS disease in the experimental autoimmune encephalomyelitis (EAE) mouse model. EAE was induced in female C57BL/6 mice and 25-day treatment with anti-Caspr-, PEG- and exosome combined GNPs was evaluated. Histopathological examination of spinal cord, regulatory T cells as well as inflammatory pathway including IFN-γ and IL-17 and mir-326 were investigated. The results showed the severity of MS symptoms was significantly decreased in all treated groups. Histological examination of the spinal cord indicated the reduced demyelination and immune cell infiltration. Besides, regulatory T cells were significantly increased following all treatments. Remarkably, the cytokine levels of IFN-γ and IL-17 as well as mir-326 is altered in treated groups. Taken together, the obtained findings demonstrate that the administration of anti-Caspr-, PEG- and exosome combined GNPs can be considered a potential treatment in MS disease.

Introduction

Multiple sclerosis (MS) is an autoimmune neurodegenerative disorder of the central nervous system (CNS) which still is increasing worldwide (1). Experimental autoimmune encephalomyelitis (EAE) is commonly used as the best animal model for CNS inflammation and demyelinating diseases (2). It has recently been shown that T cell-mediated inflammation plays a dominant role in the pathogenesis of MS (3). Different environmental factors may disrupt the peripheral tolerance of myelin-specific T cells in genetically susceptible individuals (4). In general, regulatory T cells (Treg) are one of the key elements in maintaining the balance of immune responses in a variety of autoimmune disorders as well as MS disease (5). Several reports have demonstrated that pathogenic helper T (Th) lymphocytes, especially Th1 and Th17 are major players in CNS inflammation and MS development (6–8). Moreover, pro-inflammatory cytokines including interleukin 1-beta (IL-1β), IL-12, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-17, and IL-22 are highly contributed to the MS progression (9). Therefore, aberrant pathogenic T cell responses, as well as impaired Treg cell populations most likely, underlies the MS immunopathogenesis.

MicroRNAs (miRNAs) are small regulatory non-coding RNAs that play an important role in the regulation of immune responses (10). Notably, dysregulation of miRNAs is strongly associated with neurodegenerative diseases (11). Based on previous studies miR-155 and mir-326 are involved in MS development through Th1/Th17 differentiation (12, 13).

Despite available immunomodulatory medications for MS such as Glatiramer acetate, discovering an effective and safe management strategy remains a subject of debate. Interestingly, electrical stimulation

is an important factor in axon growth and neuron survival (14). Thus, the electrically conductive materials can be helpful in nerve regeneration through the construction of nerve guidance channels (15). Gold nanoparticles (GNPs) also known as colloidal gold, are small water-soluble particles with a diameter of 1 to 100 nm in size (16). GNPs play a critical role in the improvement of axonal myelination and neuronal repair due to electrical stimulation characteristics (17). Hence, GNPs are considered a potential non-cytotoxic tool for the treatment of inflammatory diseases as well as neurodegenerative disorders (18–20). Contactin-associated protein (Caspr) is a transmembrane protein that appears on the neuronal surface following demyelination (21). Conjugation of GNPs with anti-Caspr antibody potentially targets unsheathed neurons by which may prevent the deposition of GNPs in other tissues, especially the kidneys.

Polyethylene glycol (PEG) is a synthetic biocompatible compound that can be used as a membrane adhesive by induction of cell membrane fusion (22). Furthermore, PEG additives result in the stability enhancement of biopharmaceutical proteins (23). Surprisingly, PEG rapidly repairs neuronal membranes and inhibits the production of free radicals after acute spinal cord injury in the animal model (24). Moreover, it has indicated that PEGylated GNPs can reduce inflammation and microglia response and improve myelin regeneration (17). On the other hand, exosomes are extracellular vesicles (30 to 100 nm in diameter) that are released from all eukaryotic cells through different pathways (25). Besides, exosomes can be used as molecules to transport different drugs and molecules (26). Recently, it has recently been shown that nanoparticles pass through the blood-brain barrier and are transported to the brain with the help of exosomes (27).

Hence, the aim of this study was to investigate the effect of anti-Caspr-, PEG- and exosome combined GNPs in comparison to Glatiramer acetate as a selective treatment of MS disease in the experimental autoimmune encephalomyelitis (EAE) mouse model by assessing histopathological and clinical scores.

Materials & Methods

Animals

Female C57BL/6 mice (n = 36, 8–12 weeks old) were purchased from Pasteur Institute, Tehran, Iran. All mice were housed at room temperature (RT) with a relative humidity of 40% and had free access to water and pelleted diets. Experiments were conducted based on the NIH Guideline for the Care and Use of Laboratory Animals. The research proposal was approved by the ethics committee of the Iran University of Medical Sciences (code: IR. IUMS.FMD.REC.1398.431).

Synthesis of gold nanoparticle (GNP) products

Synthesis and characterization of GNPs:

GNPs of 20 nm were synthesized according to the citrate reduction of HAuCl4 described by Frens, et al. (28). Concisely, 25 mL of 1% HAuCl4 solution (Sigma Chemical Co. Philadelphia, PA) was mixed under

continuous stirring. After reaching the boiling temperature, 1 mL of 1% trisodium citrate (Merck, Darmstadt, Germany) was added. A few minutes later, the color mixture first turned to dark violet then to deep pink which confirmed the formation of stable GNPs of 20 nm in size. Afterward, the mixture was boiled for an additional 5 min, then cooled at RT and stored at 4°C until use. The shape and size of synthesized GNPs were determined by transmission electron microscopy (TEM). Also, dynamic light-scattering (DLS) was used to evaluate the hydrodynamic diameters.

Synthesis of GNP- conjugated to anti- Contactin associated protein (Caspr)

The 0.5 mM EG6 -COOH and the 0.5 mM EG3 -OH solutions were mixed at a ratio of 1:10. One mL of the mixed solution was added to 9 mL of HAuCl4 colloidal suspension and stored for 25 h at RT. Then the mixed solution was centrifuged for 50 min at 14,000 RPM to separate the unbound and carboxylated GNPs and formation of a carboxylated self-assembled monolayer on GNPs. The pellet was resuspended in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), which was repeated two times to remove the unbound GNPs. Then 100 mL of 0.1 M EDC was mixed with 10 mL of carboxylated GNPs, followed by adding 100 µl of 0.1 M NHS to activate the GNP solution as a coupling agent. The NHS-terminated GNPs covalently combined with the side-chain amino groups on the protein surfaces. After 10 min, 1 mL of 100 g mL−1 anti-Caspr monoclonal Ab was added to the EDC/NHS-activated GNP solution. After 24 h, the solution was centrifuged for 50 min at 14 000 RPM to remove the unbound anti-Caspr mAb. Finally, the obtained anti-Caspr mAb-conjugated GNPs (GNPs–caspr mAb) were resuspended in 5 mL of 0.1 M PBS (pH 7.4) (29).

PEGylation of GNPs

To prepare an aqueous polyethylene glycol (PEG) solution (MW 1500, 30% w/v in PBS), 30 g of PEG powder (Sigma-Aldrich, Philadelphia, PA) was dissolved in 100 mL PBS. In order to PEGylation, 20 µL of PEG solution was added to each 1 mL GNP solution which was mixed on a magnetic stirrer overnight. To concentrate the PEGylated GNPs and remove any free ligand, the mixture was centrifuged at 14,000g for 30 min and then the pellet is resuspended in PBS.

Isolation and characterization of exosomes:

Exosomes were purified from the mouse serum using a Total Exosome Isolation kit (EXOCIB, Iran) according to the manufacturer's protocol. Briefly, serum was centrifuged at 3000 RPM for 20 min to remove cellular debris. The supernatant was transferred to a new conical tube and the heated reagent-A was added as a 1:5 ratio. Following the incubation overnight at 4°C, the mixture was centrifugated at 3000 RPM for 40 min and the supernatant was discarded. The pellet of the exosomes resuspend with reagent-B and the total protein concentration was measured for exosome quantification using the Bradford method (30). The quality of isolated exosomes was assessed by western blot using a primary antibody (mouse anti-CD63 antibody, concentration of 100µg/mL, (Abcam, USA) and secondary HRP-conjugated anti-mouse IgG, concentration of 200µg/mL (Santa cruz, USA). Also, TEM and DLS were done

to confirm the presence of isolated exosomes (Fig. 1). Finally, 4µg (≈ 8 x 10^9) exosomes were incubated with 10mL of 20nm GNPs for 3h at 37°C and then centrifuged and washed.

Induction and evaluation of EAE

The EAE was induced in female C57BL/6 mice by the “Salari Institute of Cognitive and Behavioral Disorders” as previously described (31). Following a ketamine/xylazine (Merck, Germany) anesthesia, all animals were immunized according to the previous protocol described by Aghaie T, et al. (17). After 48 h of the immunization, all animals have received intraperitoneal (IP) pertussis toxin (Sigma-Aldrich, P7208). Clinical signs and bodyweight of mice were assessed daily by a blind observer. Symptoms were scored according to Table 1.

Table 1
Grading system for clinical assessment of EAE

Score	Clinical signs
0	No symptoms
1	Partial loss of tail tonicity
2	Complete loss of tail tonicity
3	Flaccid tail and abnormal gait
4	Hind leg paralysis
5	Hind legs paralysis with hind body paresis
6	Hind and foreleg paralysis
7	Death

Study Design:

Mice were randomly divided into six groups and six animals were included in each group. The healthy control group (Healthy Control) is not EAE induced and received only PBS. The EAE control group (EAE Control) was EAE induced and received PBS. The third group (GNPs-Caspr mAb) is EAE induced and treated GNPs conjugated with anti-Caspr antibody and polysorbate 80. The EAE induced and treatment GNPs coated with PEG and polysorbate 80, considered a fourth group (GNPs-PEG). The EAE induced and treatment with GNPs, exosome, and polysorbate 80 (GNPs-Exosome). The EAE induced and treatment with the Glatiramer acetate (GA) drugs (GA group). Keeping in mind that polysorbate 80 (Merck, Germany) was used for the component transportation across the blood–brain barrier (BBB). In this study, 100µl of PEGylated-, Casper- conjugated and exosome-labeled GNPs with a concentration of 200µg/mL was used in all mice. All the animals received treatments through IP injection 1 day in between from day 3 to day 24 post-immunization (in total 12 injections). GA treatment (150µg/mouse by subcutaneous injection) started at day 3 after EAE induction. Daily injection of GA continued until the end of experiment.

25 days after EAE induction, the mice were anesthetized and spleens were removed and single-cell suspension of splenocytes were obtained. In brief, the spleen was perfused and the cells were passed through the cell strainers of 70 and 40µm. The cells were centrifuged at 300g for 10min, and the supernatant was discarded. To lysis the red blood cells, the splenocytes were incubated with 10mL ACK 1X (ammonium-chloride-potassium) lysing buffer for 5min at 4°C. The mixture was centrifuged at 300g for 5min and the supernatant was removed. After washing once with RPMI-1640 medium, the reminding cell suspensions were the spleen mononuclear cells. Then, all animals were sacrificed according to the ethics protocol and the spinal cord was removed for further histopathological studies.

Detection of regulatory T cells by flow cytometry analysis

The treatment effect on Treg cells was determined by flow cytometry technique using mouse regulatory T cell staining kit (eBioscience, San Diego, CA). In order to detect the cell surface markers, 1×10^6 spleen cells from all treatment groups were stained with the following fluorochrome labeled monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (clone RM4) and phycoerythrin (PE)-conjugated anti-mouse CD25 (clone PC61.5) antibodies at 4°C for 30 min. Cells were fixed and permeabilized with fixation/permeabilization buffer for the evaluation of FoxP3. Then the cells were incubated in this buffer containing (APC)-conjugated anti-mouse/Rat Foxp3 (clone FJK16s) antibody at 4°C for 30 min. Ultimately, all samples were washed and suspended in kit-provided staining buffer and then analyzed in a Mindray (BriCyte E6, China) with associated FlowJO 10 software. A minimum of 50000 events in the lymphocyte gate was collected per sample.

Histopathology

The spinal cord was isolated and horizontal paraffin-embedded sections (5µm thick) of the spinal cords were stained by H&E and Luxol Fast Blue Staining for evaluating the cell infiltration and myelination, respectively. Slides were assessed in a blinded manner (by analysis of three fields) and histological scores were determined as in our previous study.

RNA Extraction, Quantitative real-time PCR (qPCR), and MicroRNA Quantification

The spleen cells were isolated from EAE mice treated with GNPs. Large and small (total) RNA was extracted from splenocytes using GeneAll^R RiboEx Total RNA extraction kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. The quantity and quality of RNA were evaluated by a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The cDNA synthesis was performed from large RNA in the thermal cycle using miScript Reverse Transcription kit (Parstoos, Iran) according to manufacturer's protocols for the treatment effect on mRNA expression of IL-17 and IFN-□ cytokines were determined by qPCR. HGPRT was considered as a housekeeping gene. The specific forward and reverse primers for the evaluation of IL-17 and IFN-□ genes expression were designed by NCBI primer blast (Table 2). The specific forward and reverse primers of miR-326-3p were purchased from Pars Genome, Tehran, Iran. Then the qPCR was carried out using the miScript SYBR Green PCR Kit (Pars Genome, Iran) and Qiagen Real-Time PCR system (Applied Biosystem). The RNU6 gene was used as an internal control

for normalization. The threshold cycles (CT) were normalized and the relative expression levels were calculated using the 2-ΔΔct method.

Table 2
The sequence of primers which are used in this study.

Gene Name	Sequence		Product length (bp)
Interleukin- 17A (IL-17a)	Forward	5′-CAGACTACCTCAACCGTTCC-*3′*	120
	Reverse	5′-CCTCCGCATTGACACAGC-*3′*	
Interferon- gamma (IFN-γ)	Forward	5′-CACACCTGATTACTACCTTCTTC-*3′*	180
	Reverse	5′-AGCAGCGACTCCTTTTCC-*3′*	
Hypoxanthine guanine phosphoribosyl transferase (HGPRT)	Forward	5′-TCCCAGCGTCGTGATTAG-*3′*	138
	Reverse	5′-CGAGCAAGTCTTTCAGTCC-*3′*	

Statistical analysis

Statistical analysis was performed using Prism (GraphPad) Software (version 8.3.0). Data are presented as Mean ± SEM and p values < 0.05 were statistically considered as the significance level. The normal distribution of data was examined by the Kolmogorov-Smirnov test. ANOVA test was used to compare the groups and analysis of the data. The Friedman test is used to evaluate the clinical score and body weight of all groups.

Results

Characterization of GNPs:

TEM and DLS are respectively used to determine the shape and size distribution of synthesized GNPs (Fig. 2). As Fig. 1b shows, the mean size of over 90% of GNPs was ~ 20 nm in diameter.

Improved clinical Scores and reduced Body Weight in treated mice

Body-weight and clinical signs of all mice were monitored, daily. The obtained results have shown an improvement in the behavioral and neurological symptoms of mice that are treated with GNPs. Also, a significant weight loss was observed over the 25 days period in the EAE control group. The treatment with GNPs increases the body-weight of mice in all treatment groups (Fig. 3). Clinical manifestations of EAE were observed about 10 days after immunization in the untreated group (EAE ctrl) and between days 14 and 15 in the treated groups (Fig. 4). Moreover, the severity of clinical signs had significantly decreased in the treated mice in comparison to the EAE control group ($P \leq 0.05$). Treatment with GNPs-Caspr mAb,

GNPs-PEG, and GNPs-exosome reduced the incidence of disease. However, there was no significant difference in clinical scores between the GNP-treated groups (Table 3).

Table 3
Clinical features of EAE in the administration of anti-Caspr-, PEG-, exosome combined GNP and Glatiramer acetate

Groups	Incidence (sick/total)	Day of onset	Peak Day Score	Maximum mean clinical score	Cumulative disease index CDI
EAE Control	6/6 100%	10	19	2.95 ± 1.28	242
Glatiramer Acetate	5/6 83%	14	18	0.55 ± 0.50	56
GNPs + Caspr	4/6 66%	15	19	0.37 ± 0.47	19
GNPs + PEG	5/6 83%	15	17	0.41 ± 0.60	35
GNPs + Exosome	5/6 83%	14	18	0.50 ± 0.61	43

GNPs effects on demyelination/inflammation in the spinal cord of EAE mice

The histopathological analysis of the spinal cord from all treated mice was carried out to evaluate the underlying reasons for the improved clinical manifestations. The infiltration of mononuclear cells, as well as the myelin loss, were characterized in order to evaluate inflammation and demyelination, respectively. The semi-quantitative results of the histopathological features demonstrated a remarkable infiltration of inflammatory cells into the spinal cord and local demyelination in the EAE control group. Furthermore, declined demyelination occurred due to the reduced inflammatory cell infiltration to the spinal cord of all GNPs-treated mice. Interestingly, histopathological evaluation of mice showed that the treatments with GNPs modulate the severity of EAE (Fig. 4).

Discussion

MS is viewed as a more frequent chronic immune-mediated demyelinating disease of the CNS worldwide. Despite the notable progress in the treatment of MS, it still remains a major neurological disorder at a global level. Thus, the discovery of a novel effective therapeutic method in order to the management of disease through reduction MS complications is perceptible. This study provides new insight into the potential role of anti-Caspr, PEG- and exosome combined GNPs in the treatment of EAE that is considered a prototype of MS disorder.

According to recent researches, it is reasonable to perceive that GNPs have an anti-inflammatory effect which can be considered as a therapeutic agent in neuro-immunological disorders (32). In general,

previous studies have shown that GNPs play an important neuroprotective role through the improvement of clinical symptoms in mice with autoimmune disorders including Parkinson's disease, Alzheimer's disease, and EAE (17, 33–35). However, targeted therapy emerges as the best strategy regarding the treatment of MS disease and animal model of EAE. Based on the MS pathogenesis, GNPs combined with an anti-Caspr mAb can be considered a novel approach to target neuronal damage and unsheathed myelin. This study highlights three different approaches in the treatment of the EAE animal model. Interestingly, this study has demonstrated that the treatment with GNP- conjugated with anti-Caspr antibody improved the clinical symptoms in EAE mice ($P \leq 0.05$). Recently, in-vivo studies have reported that PEG treatment has a major influence on the reduction and improvement of clinical signs in EAE animal models (36). Moreover, Aghaie, T. et al have depicted that EAE treatment by PEG significantly decreases the severity of MS symptoms (17). Hence, PEGylation of GNPs could open a new horizon in the management and limitation of MS disorder.

On the other hand, exosomes are extracellular double-membrane microvesicles that contain different biomolecules particularly microRNAs with immunomodulatory effects (37). Approximately 30% of exosomes isolated from serum were originated from neuronal cells (38). Therefore, a combination of the exosome and GNPs could improve the application of neurodegenerative disease treatment.

Surprisingly, the results of this study showed that treatment of EAE mice with Caspr-, PEG- and exosome combined GNPs reduced the inflammation and help the nerve cell repairment which subsequently results in the improvement of clinical symptoms. Besides, the obtained results have indicated that there is no significant difference between the three treatment groups ($P \leq 0.05$). Remarkably, no significant difference was observed between the effects of three different treatments in comparison to the glatiramer acetate as a selective treatment for MS. Furthermore, all treatments result in a significant reduction in weight loss among EAE mice ($P \leq 0.05$). Recently, disruption in T cell polarization, as well as Treg cell reduction, have been shown in MS disease. In this sense, the development of regulatory T cells may be useful to inhibit the autoreactive T cells activation and modulation of autoimmune disorders. The results show a significant increase in the number of Treg lymphocytes in the spleen of EAE mice treated with anti-Caspr-, PEG- and exosome combined GNPs. Besides, regulatory T cells reduce inflammation; In addition, previous studies have shown that the Th1/Th17-mediated inflammatory pathways play an important role in the pathogenesis of MS disease. It is reasonable to presume that proinflammatory cytokines including INF-γ and IL-17 significantly higher in MS patients compared to normal subjects. The obtained results indicated that anti-Caspr-, PEG- and exosome combined GNPs reduce the proinflammatory cytokine production.

On the other hand, Du Ch, et al. have demonstrated mir-326 promotes the Th17 differentiation that results in the severity of MS disease (39). Moreover, the expression level of mir-326 in active MS plaques is significantly upregulated in comparison to healthy individuals (40). The present study also showed that the level of mir-326 in the EAE animal model was significantly higher than in the healthy control group ($P \leq 0.05$). Interestingly, the treatment of mice using anti-Caspr mAb conjugated GNPs significantly reduced

mir-326 expression. Also, administration of PEG- and exosome combined GNPs remarkably influence mir-326 expression in EAE treated mice.

Taken together, administration of anti-Caspr-, PEG- and exosome combined GNPs can be considered a potential treatment in MS disease due to induction of Treg development and reduction in proinflammatory cytokines especially IL-17 and IFN-γ.

Declarations

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Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

We declare that there is no conflict of interest in publication of this contribution.

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Figures

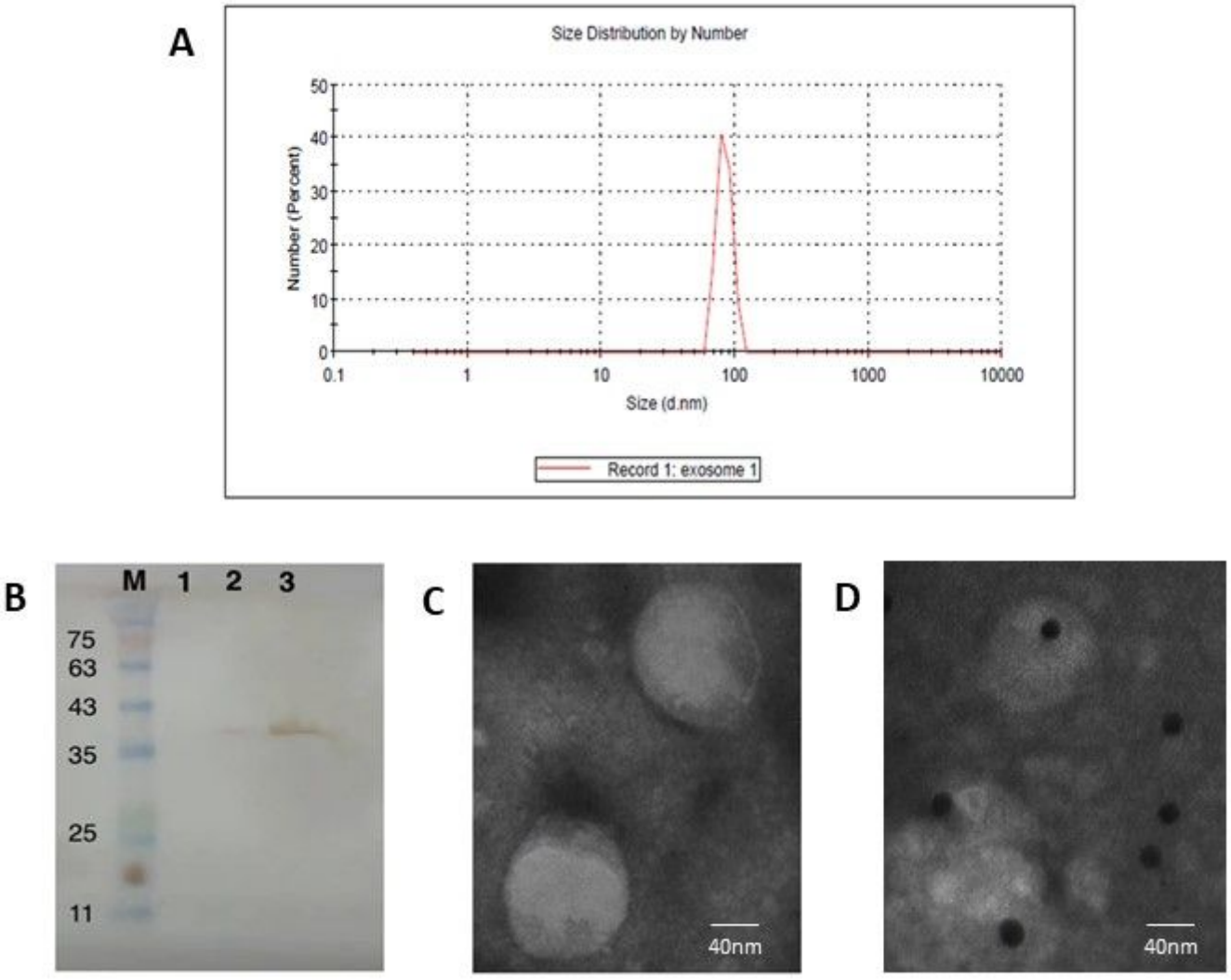

Figure 1

Characterization of the purified exosome. (A) Exosome size distribution by dynamic light scattering. (B) Western blot analysis of extracted exosome from the mouse serum. Lane 2 and 3 are shown two different concentrations of 16.8 and 168 µg/ml, respectively. Lane 1 is a PBS, as a negative control (C) Transmission electron microscopy (TEM) of exosomes isolated from mouse serum. (D) Transmission electron microscopy (TEM) of exosomes with GNPs.

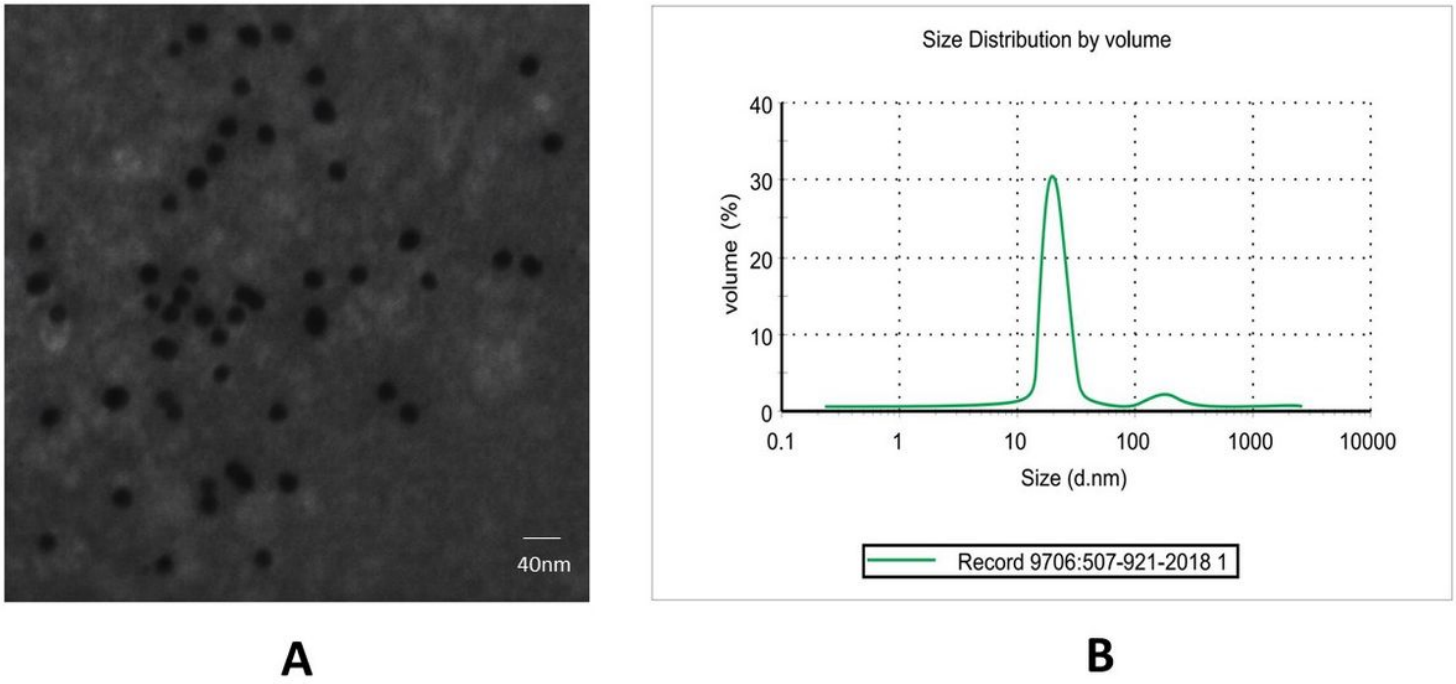

Figure 2

Characterization of the gold nanoparticles (GNPs). (A) TEM image of GNPs and (B) GNP size distribution by dynamic light scattering.

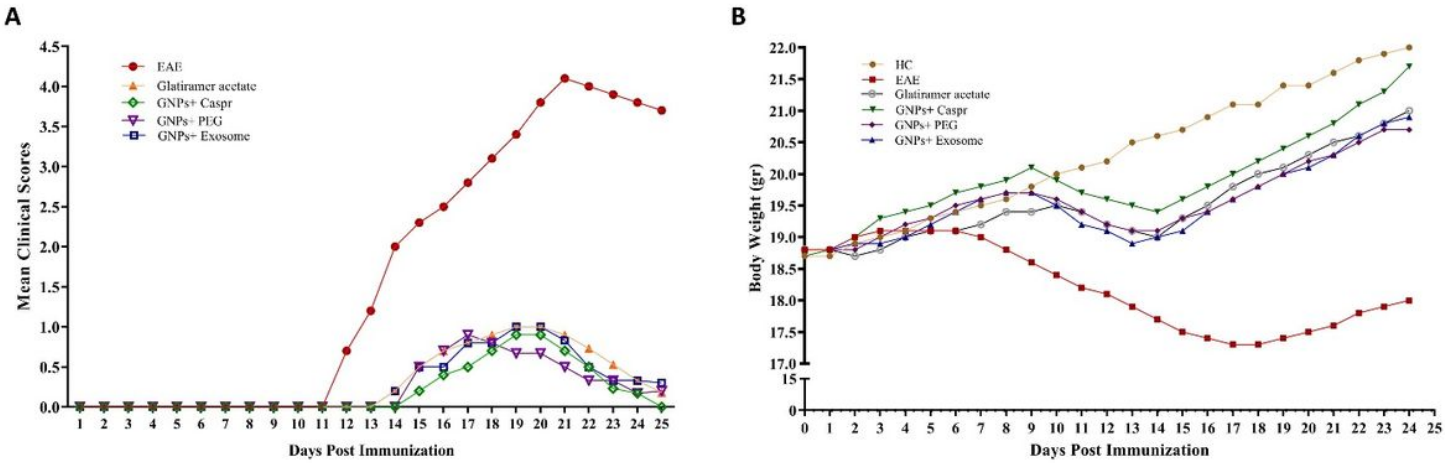

Figure 3

Clinical score and bodyweight of mice after treatment with anti-Caspr-, PEG-, exosome combined GNPs and Glatiramer acetate in comparison to the EAE control. (A) A remarkably reduced severity of clinical scores in all treated groups compared to EAE control ($P < 0.001$). (B) A significant increase in the mean body weight changes is shown in all groups compared to EAE control ($P < 0.001$).

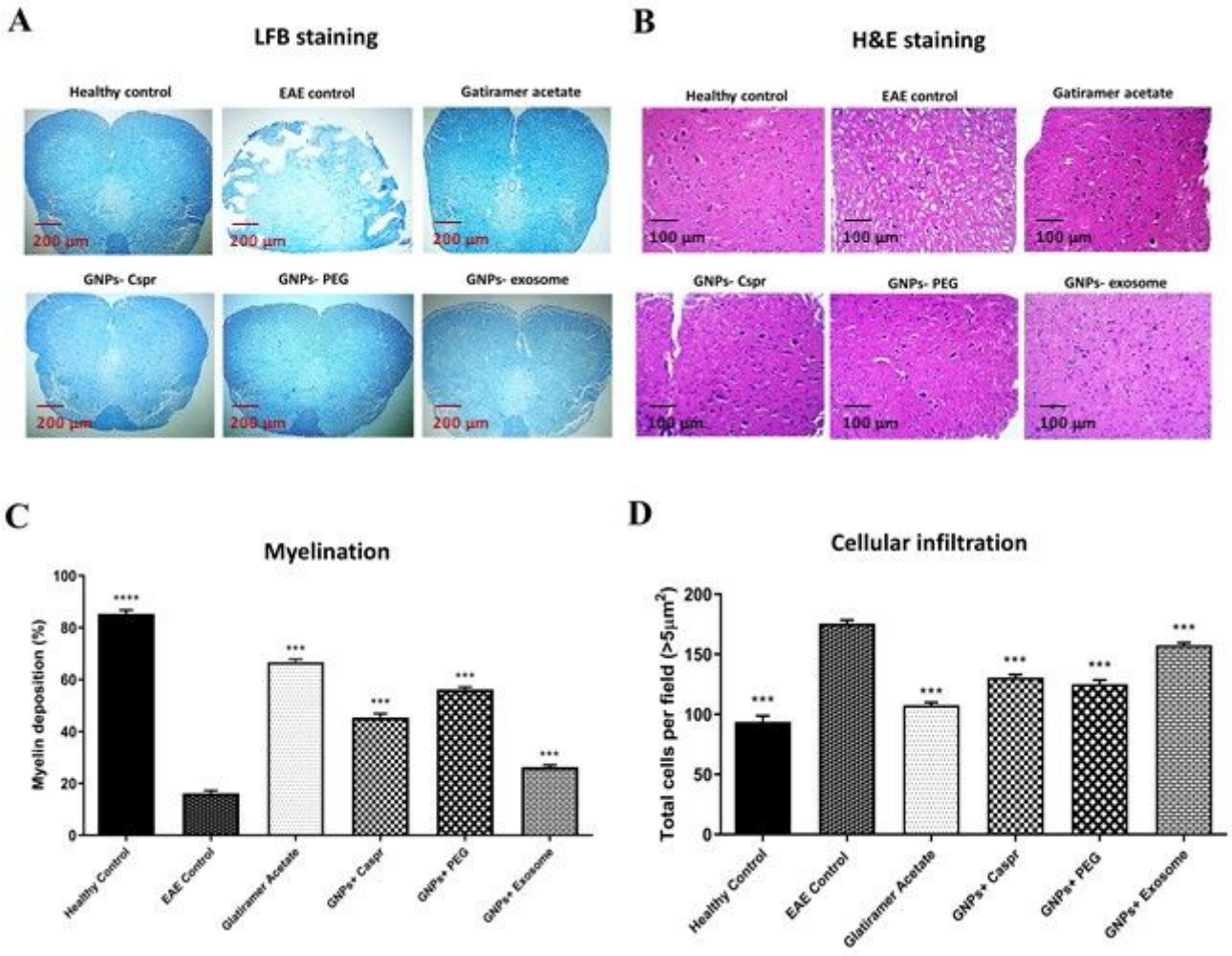

Figure 4

The effect of treatment with anti-Caspr-, PEG-, exosome combined GNP and Glatiramer acetate on pathology associated with EAE, in comparison to the EAE control. Spinal cords from each mouse (collected on Day 25 post-immunization) were fixed, embedded in paraffin, sections (5-mm) were prepared and the tissues were then stained with (A) Luxol fast blue staining to assess the extent of demyelination and (B) H&E staining to permit enumeration of infiltrating cells. Histological features were scored semi-quantitatively as described in the Methods section. (C) LFB staining (transverse sections) shown reduced demyelinated lesions in treated groups compared with no treatment group. (D) Reduced inflammatory infiltration in treated groups compared with EAE control group. The Values shown are mean ± SEM. The significant level was obtained using the one-way ANOVA test followed by a post hoc Tukey's test.

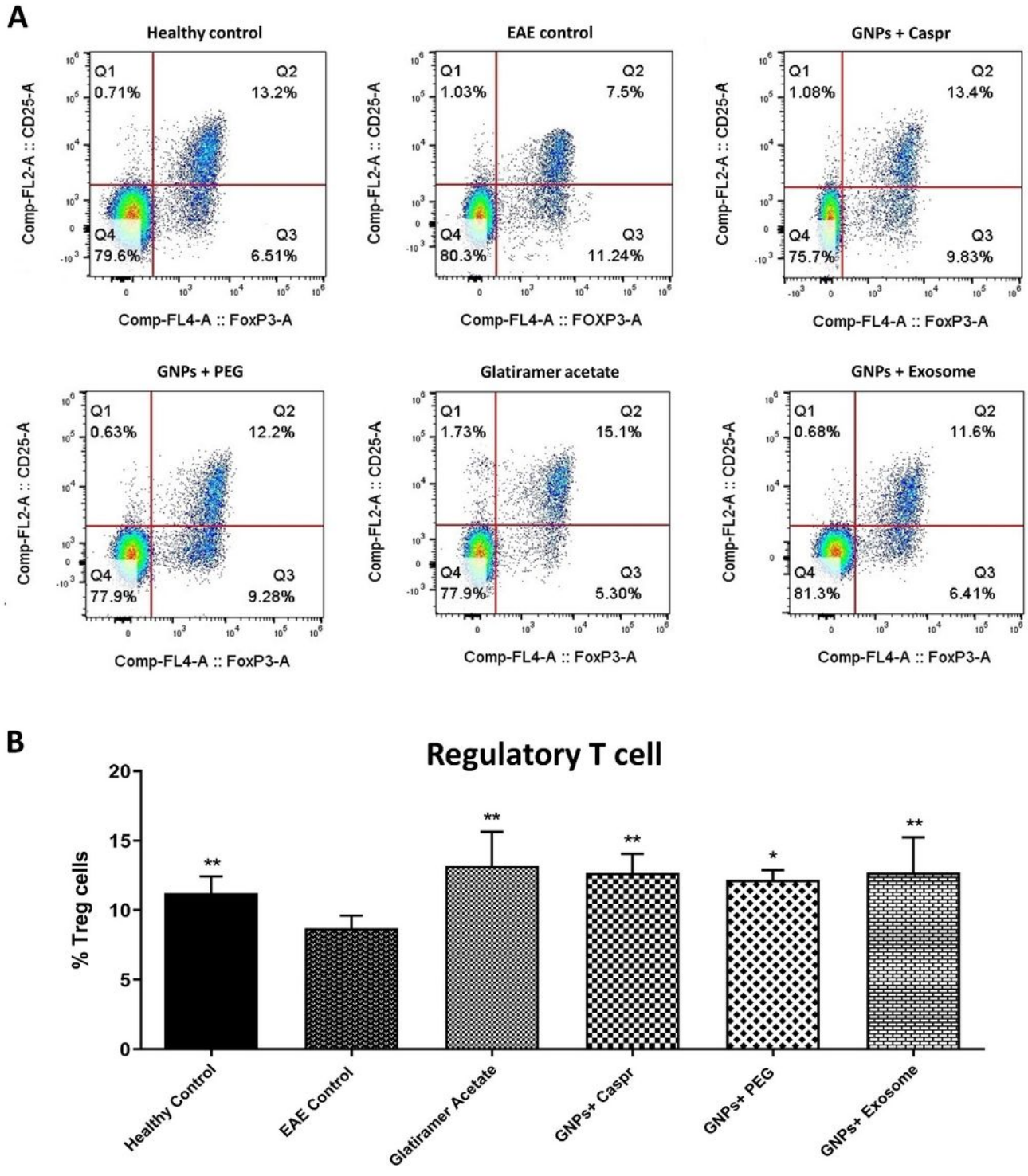

Figure 5

Treatment with anti-Caspr-, PEG-, exosome combined GNP and Glatiramer acetate administration promoted the frequency of Treg cell population in comparison to the no treatment group. Splenocytes were stained with anti-CD4 FITC, anti-CD25 PE, then cells were fixed and permeabilized and stained with anti-Foxp3 APC. Lymphocytes were gated by forward and side scatter. Lymphocytes were then gated on

the CD4+population and the quadrant marker was then set on CD25 PE vs. Results are expressed as mean ± SEM. *P < 0.05,**P < 0.01,***P < 0.001, and****P < 0.0001.

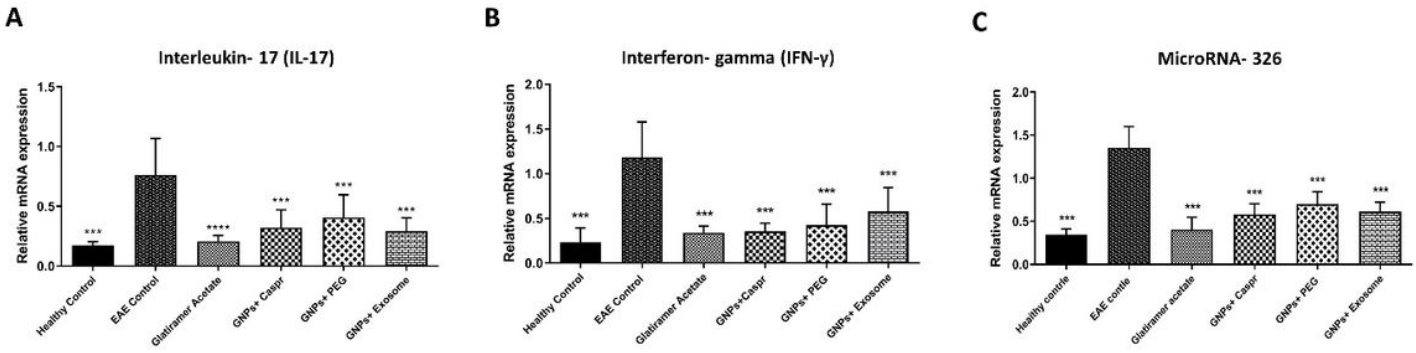

Figure 6

Effects treatment with anti-Caspr-, PEG-, exosome combined GNP and Glatiramer acetate on the expression levels of pro-inflammatory cytokines and miR-326. (A, B) The figure shows that all treatments led to the suppression of pro-inflammatory cytokines. (C) miR-326 is down-regulated in splenocytes in comparison to the no treatment group. Splenocytes were isolated from mice in all groups, RNA was extracted and the expression levels of miR-326 were quantified by RT-PCR. The values are means of triplicates at each point and the error bars represent SEM. Statistical analysis was performed by one-way ANOVA followed by a post hoc Tukey's test. *P < 0.05, **P < 0.01, ***P < 0.001, and****P < 0.0001.